



Office de la Propriété
Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2096532 C 2002/12/31

(11)(21) 2 096 532

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

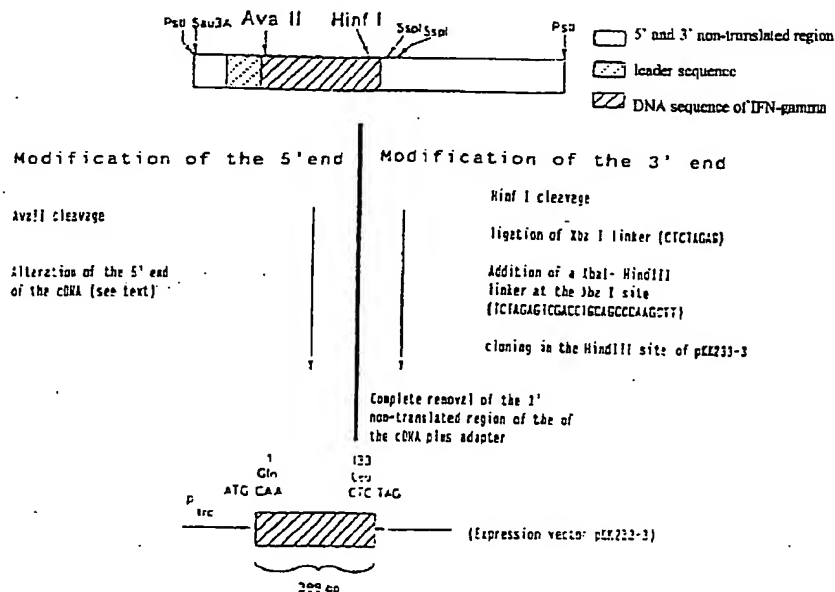
(86) Date de dépôt PCT/PCT Filing Date: 1991/11/14
(87) Date publication PCT/PCT Publication Date: 1992/05/29
(45) Date de délivrance/Issue Date: 2002/12/31
(85) Entrée phase nationale/National Entry: 1993/05/18
(86) N° demande PCT/PCT Application No.: DE 1991/000912
(87) N° publication PCT/PCT Publication No.: 1992/008737
(30) Priorité/Priority: 1990/11/19 (P 40 36 856.4) DE

(51) Cl.Int.⁵/Int.Cl.⁵ C12N 15/23, A61K 38/21, C07K 14/57, C07K 1/22
(72) Inventeurs/Inventors:
SLODOWSKI, OTTO, DE;
BOHM, JOACHIM, DE;
OTTO, BERND, DE
(73) Propriétaire/Owner:
FRAUNHOFER-GESELLSCHAFT ZUR FORDERUNG
DER ANGEWANDTEN FORSCHUNG E.V., DE
(74) Agent: MACRAE & CO.

(54) Titre : NOUVEL INTERFERON GAMMA RECOMBINANT HUMAIN
(54) Title: NEW HUMAN RECOMBINANT GAMMA-INTERFERON

Construction of an expression plasmid for expression of IFN-gamma C-10L

Human IFN γ C-10L cDNA cloned in the PstI site of pBR322



(57) Abrégé/Abstract:

The invention concerns a new mutant of γ -IFN. This new polypeptide contains 134 amino acids. Amino acids 1 to 132 are the same as those of natural γ -interferon. The first amino acid, methionine, in the zero position is also present, as has been demonstrated by protein sequencing. The amino acid in position 133 is leucine instead of glutamine. The invention also concerns DNA sequences and plasmid DNA (DSM 6238) which codes for this new polypeptide. The invention further concerns the use of the polypeptide as a drug as well as its use as a fine-chemical reagent for in vitro experiments.

Canada

<http://opic.gc.ca> • Ottawa-Hull K1A 0C9 • <http://cipo.gc.ca>

OPIC • CIPO 191

OPIC CIPO

BEST AVAILABLE COPY

2096532

Title: NEW HUMAN RECOMBINANT γ INTERFERON

Abstract

The invention concerns a new mutant of γ -IFN. This new polypeptide contains 134 amino acids. Amino acids 1 to 132 are the same as those of natural γ -interferon. The first amino acid, methionine, in the zero position is also present, as has been demonstrated by protein sequencing. The amino acid in position 133 is leucine instead of glutamine. The invention also concerns DNA sequences and plasmid DNA (DSM 6238) which codes for this new polypeptide. The invention further concerns the use of the polypeptide as a drug as well as its use as a fine-chemical reagent for *in vitro* experiments.

2096532

1

New human recombinant gamma-interferon

Technical field

The invention relates to a new mutant of gamma- interferon, the DNA sequences and plasmid DNA that code for this mutant gamma-interferon and the use of the mutant interferon for medicinal purposes.

State of the art

Interferons have been divided into three classes, namely alpha-interferon, beta-interferon and gamma-interferon.

In particular gamma-interferon has recently gained importance due to its use as a therapeutic agent. This was most of all due to the successful production of recombinant gamma-interferon by genetic engineering (recombinant gamma-interferon)

Because of its systemic application in medicine, however high concentrations of interferon are used . These high concentrations place high demands on the formulation of the interferon, and in addition can contribute to antigenicity.

The use of gamma-interferon as a therapeutic agent would therefore be more attractive if a mutant demonstrating higher activity was available so that lower concentrations could be used in therapy. Furthermore, it would be desirable that this mutant interferon could be expressed at a high rate.

It is an object of this invention to provide a new mutant of gamma-interferon that shows an increased activity relative to previously described gamma-interferon with 144 amino acids. It is a further object of the present invention to provide DNA sequences and plasmid DNA that code for the mutant gamma-interferon. It is also a further object of the present invention to provide methods that permit the production of the mutant gamma-interferon in the highest possible yields.

Description of the invention

The foregoing objects are solved by providing DNA sequences and plasmid DNA coding for an amino acid sequence that results in a recombinant gamma-interferon showing a substantially higher activity. This new polypeptide (referred to in the following text as gamma-interferon C-10L) contains 134 amino acids. Amino acids 1 to 132 correspond to those of natural gamma-interferon. The first amino acid, in position 0, is additionally present and was determined by amino acid sequencing. The amino acid in position 133 is leucine instead of glutamine. The complete DNA and protein sequence is given in Figure 1. At the same time a method is described that allows the mutant gamma-interferon to be produced in high yields. There is also the purification through a so-called "batch-protocol".

Surprisingly, it was found that the gamma-interferon C10L had a 4-fold higher activity than the complete 143 amino acid gamma-interferon. Gamma-interferon C-10L has, in addition, a 24-fold higher activity, than normal gamma-interferon, as anti-proliferative activity, on human WISH cells. In contrast to previously known gamma-interferon, and because of its higher activity and expression rate, this shortened form makes, available for the first time, a gamma-interferon that allows lower and more target oriented doses to be used for therapeutic purposes. Because of the high expression rate it is further possible to use this gamma-interferon as a fine chemical for in vitro experiments e.g. as a standard for interferon level measurements.

The detailed description of the new mutant and its production are described below.

The production of gamma interferon C-10L is achieved by the cleavage of part of the gene and placing it in a plasmid DNA behind a regulatory region, followed by transfection of bacterial cells using this DNA. In a further step gamma-interferon-C10L is concentrated using a cation exchange process and as second step it undergoes high purification.

By way of example, the construction of the plasmid DNA (deposit number DSM 6238) that codes for the gamma-interferon C-10L is described below.

The starting material used a cDNA that was produced by standard methods from human cells. This cDNA was sequenced and has a length, without the poly-A, of 1194 base-pairs (bp). It contains the entire coding region as well as 5' and 3' non-translated sequences. The nucleotides 182 to 574 were used for the construction of an expression plasmid.

The 5' non-translated region, (1-109) as well as the leader sequence of the protein, was removed with restriction endonuclease AvaII (recognizing the sequence GGA/TCC, 181-185). The initiation codon ATG (for Met) as well as the codon for the first amino acid were attached to the 5'-end of the cDNA using a synthetic DNA fragment (commercially available linkers). In this way the natural codon CAG (for Gln) was replaced by CAA (also for Gln). The above mentioned technology is "state of the art".

The 3'-untranslated region, as well as a part of the coding region were removed by cleavage with HinfI (recognition sequence GANTC, 571-575). The ligation of a linker with an XbaI (CTCTAGAG) recognition site provides the codons for amino acid 133 (Leu) as well as the stop codon (TAG). The shortened cDNA was inserted into an expression vector using an adapter sequence.

To express gamma interferon in E. coli, plasmid pKK233-2, constructed by E. Amman and J. Brosius (GENE 40, (1985) 1893) was used. It possesses the inducible trc-promoter, a multiple cloning site containing the start codon (ATG) as well as terminators for RNA polymerase.

This plasmid DNA (deposit number DSM 6238) constitutes the starting material for the production of gamma-interferon C-10L.

The gamma-interferon C-10L was expressed at a rate of 30% of the total protein in JM 105 bacterial cells. Over 90% of the gamma-interferon C-10L was in the form of insoluble inclusion bodies.

For the purification of the gamma-interferon, the bacterial cells were broken open and the inclusion bodies were freed of soluble bacterial proteins by repeated washing. Cell breakage was preferably mechanical; in particular ultrasonics were used. The inclusion bodies and thereby the gamma-interferon was solubilized by a denaturation step using guanidine chloride. The gamma-interferon was brought back to a biologically active form in a renaturation step by dilution into phosphate buffer. In this way greater than 90% pure gamma-interferon was concentrated via a cation exchange process in a "batch protocol" and further purified through a gel filtration step to a purity of more than 95%.

The term "batch protocol" is used in this invention to describe the following process: The cation exchange material was stirred into a gamma-interferon solution in such a way that the gamma-interferon was bound to the total ion exchange material, and the gamma-interferon protein concentration was not higher than about 2mg/ml of packed ion exchange material. This procedure is referred to as the "batch protocol". The gamma-interferon loaded ion exchange material was then washed with phosphate buffer in batch and the gamma-interferon then eluted in batch with a sodium chloride solution in phosphate buffer. Either CM-Cellulose™ or Affi-gel Blue™ can be used as cation exchange material.

This "batch protocol" offers decisive advantages and leads to an increased yield during the purification of 40% compared to 10% with the usual column method.

This ion exchange process is normally carried out by column chromatography; after the renaturation step in the presence of e.g. 0.2 M guanidine chloride and a phosphate buffer, the gamma-interferon is pumped onto a column of cation exchange material and binds, due to the high binding capacity of the cation exchange material for the gamma-interferon, with a

correspondingly high concentration in the upper part of the column. Because of the high concentration of bound gamma-interferon the corresponding elution yield is very low; i.e. only a small part of the bound interferon can be eluted by the corresponding salt solutions. Therefore the method according to the present invention offers decisive advantages over the state of the art.

Figure 1 shows the DNA and protein sequence of the gamma-interferon C-10L variant.

From this it can be seen that the gamma-interferon mutant C-10L contains 134 amino acids. The first amino acid (methionine in position 0) is an addition and was confirmed by protein sequencing. The amino acids 1 to 132 correspond to those of natural interferon. Amino acid 133 is leucine instead of glutamine. The correct structure of the nucleotides of the coding sequence and of the flanking regions was verified by DNA sequencing.

Gamma-interferon C-10L has a molecular weight of about 30,000 daltons under native conditions and a S-value of 2.5; i. e. it is organized in the form of a dimer. Under denaturing conditions in SDS it is a monomer having a molecular weight of about 15,000 daltons. The isoelectric point is 10.0.

The gamma-interferon C-10L has a specific antiviral activity of 8×10^7 U/mg protein (measured on human lung fibroblast carcinoma cells A549 with EMC virus). This is 4 times more active than the complete 143 amino acid gamma-interferon.

The gamma-interferon C-10L has a 24 fold higher anti-proliferative activity on human WISH cells compared to gamma-interferon.

The gamma-interferon C-10L can also be used as a therapeutic agent for the treatment of rheumatism and kidney cancer.

The gamma-interferon C-10L induces the expression of MHC class II antigens HLA-DR on human colon carcinoma cells eight fold better than does gamma-interferon. Surprisingly receptor binding of the gamma interferon C-10L to these cells is 2 fold worse than the corresponding receptor binding for gamma-interferon. The receptor binding was measured with ³²P labelled gamma-interferon in competition experiments in which ³²P labelled gamma-interferon competes with unlabelled gamma-interferon or interferon C-10L and vice versa ³²P labelled gamma interferon C-10L with unlabelled gamma-interferon C-10L or gamma interferon.

In this important characteristic, namely the receptor binding, the gamma-interferon C-10L is different from one of gamma interferons previously described in the document "Role of the carboxy-terminal sequence on the biological activity of interferon- γ ", G. Garotta et al., J. Biotechnol. 7, 1988, 199-216 which also has a 10 amino acids shortened COOH terminus but this variant has as terminal amino acid a glutamine instead of the leucine on the gamma-interferon C-10L and shows 4 fold higher receptor binding compared to gamma interferon.

The herein described gamma-interferon C-10L recombinant makes available, for the first time, a mutant gamma-interferon with enhanced activity that can be produced in higher quantities.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A polypeptide IFN-gamma C-10L having the following
amino acid sequence :

```

0
Met
ATG

1
GlnAspProTyrValLysGluAlaGluAsnLeuLysLysTyrPheAsnAlaGlyHisSer
CAAGACCCATATGTAAAAGAAGCAGAAAACCTTAAGAAATATTTTAATGCAGGTCATTCA
179

40
AspValAlaAspAsnGlyThrLeuPheLeuGlyIleLeuLysAsnTrpLysGluGluSer
GATGTAGCGGATAATGGAACTCTTTTCTTAGGCATTTTGAAGAATTGGAAAGAGGAGAGT

60
AspArgLysIleMetGlnSerGlnIleValSerPheTyrPheLysLeuPheLysAsnPhe
GACAGAAAAATAATGCAGAGCCAAATTGTCTCTTTTACTTCAAACCTTTTAAAAACTTT

80
LysAspAspGlnSerIleGlnLysSerValGluThrIleLysGluAspMetAsnValLys
AAAGATGACCAGAGCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATGAATGTCAAG

100
PhePheAsnSerAsnLysLysLysArgAspAspPheGluLysLeuThrAsnTyrSerVal
TTTTTCAATAGCAACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAATTATTCGGTA

120
ThrAspLeuAsnValGlnArgLysAlaIleHisGluLeuIleGlnValMetAlaGluLeu
ACTGACTTGAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGATGGCTGAACTG

133
SerProAlaAlaLysThrGlyLysArgLysArgSerLeu
TCGCCAGCAGCTAAAACAGGGAAGCGAAAAAGGAGTCTC TAG
574

```

2. A DNA sequence coding for a polypeptide with the following amino acid sequence :

```

0
Met
ATG

1
GlnAspProTyrValLysGluAlaGluAsnLeuLysLysTyrPheAsnAlaGlyHisSer      20
CAAGACCCATATGTAAAAGAAGCAGAAAACCTTAAGAAATATTTTAATGCAGGTCATTCA
179

AspValAlaAspAsnGlyThrLeuPheLeuGlyIleLeuLysAsnTrpLysGluGluSer      40
GATGTAGCGGATAATGGAACCTCTTTCTTAGGCATTTTGAAGAATTGGAAAGAGGAGAGT

AspArgLysIleMetGlnSerGlnIleValSerPheTyrPheLysLeuPheLysAsnPhe      60
GACAGAAAAATAATGCAGAGCCAAATTGTCTCCTTTTACTTCAAACCTTTTAAAACTTT

LysAspAspGlnSerIleGlnLysSerValGluThrIleLysGluAspMetAsnValLys      80
AAAGATGACCAGAGCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATGAATGTCAAG

PhePheAsnSerAsnLysLysLysArgAspAspPheGluLysLeuThrAsnTyrSerVal      100
TTTTTCAATAGCAACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAATTATTCGGTA

ThrAspLeuAsnValGlnArgLysAlaIleHisGluLeuIleGlnValMetAlaGluLeu      120
ACTGACTTGAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGATGGCTGAACTG

SerProAlaAlaLysThrGlyLysArgLysArgSerLeu      133
TCGCCAGCAGCTAAAACAGGGAAGCGAAAAAGGAGTCTC TAG
574

```

10

3. A plasmid DNA with the deposit number DSM 6238, coding for the following amino acid sequence :

0
Met
ATG

1 20
GlnAspProTyrValLysGluAlaGluAsnLeuLysLysTyrPheAsnAlaGlyHisSer
CAAGACCCATATGTAAAAGAAGCAGAAAACCTTAAGAAATATTTTAATGCAGGTCATTCA
179

40
AspValAlaAspAsnGlyThrLeuPheLeuGlyIleLeuLysAsnTrpLysGluGluSer
GATGTAGCGGATAATGGAACCTCTTTCTTAGGCATTTTGAAGAATTGGAAAGAGGAGAGT

60
AspArgLysIleMetGlnSerGlnIleValSerPheTyrPheLysLeuPheLysAsnPhe
GACAGAAAAATAATGCAGAGCCAAATTGTCTCCTTTTACTTCAAACCTTTTAAAAAAGCTT

80
LysAspAspGlnSerIleGlnLysSerValGluThrIleLysGluAspMetAsnValLys
AAAGATGACCAGAGCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATGAATGTCAAG

100
PhePheAsnSerAsnLysLysLysArgAspAspPheGluLysLeuThrAsnTyrSerVal
TTTTTCAATAGCAACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAATTATTTCGGTA

120
ThrAspLeuAsnValGlnArgLysAlaIleHisGluLeuIleGlnValMetAlaGluLeu
ACTGACTTGAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGATGGCTGAACTG

133
SerProAlaAlaLysThrGlyLysArgLysArgSerLeu
TCGCCAGCAGCTAAAACAGGGAAGCGAAAAAGGAGTCTC TAG

574

4. A process for preparing a plasmid DNA according to claim 3, wherein the 5' non-translated region and a part of the coding region of a cDNA with 1194 bp and 143 amino acids are removed by cleavage with a restriction enzyme, and the 3' non-translated region as well as a part of the coding region are also removed by cleavage with a restriction enzyme, and this gene is inserted into plasmid pkk233-2 and introduced into bacterial cells.
5. A process according to claim 4 wherein the bacterial cell is *E. coli*.
6. A process according to claim 4 or 5 wherein the restriction enzyme used for cleavage of the 5' non-translated region and the part of the coding region of a cDNA is restriction endonuclease *Avall*.
7. A process according to claim 4 or 5 wherein the restriction enzyme used for cleavage of the 3' non-translated region and the part of the coding region is *Hinfl*.
8. A process for preparing a polypeptide according to claim 1 comprising the following steps:
 - a) the plasmid DNA according to claim 3 is introduced into bacterial cells;
 - b) the bacterial cells, following successful expression, are lysed and the inclusion bodies obtained are freed from soluble bacterial proteins by washing;
 - c) the inclusion bodies are brought into solution by denaturation and then undergo a renaturation step; and
 - d) the gamma-interferon C-10L is purified.
9. A process according to claim 8, wherein the gamma-interferon is concentrated using a batch method via a cation exchange process using a cation exchange material and then is highly purified by gel filtration.

10. A process according to claim 8 wherein the bacterial cells are lysed by a mechanical process.
11. A process according to claim 9 wherein the bacterial cells are lysed by a mechanical process.
12. A process according to claim 10 wherein ultrasonics are used to lyse the bacterial cells.
13. A process according to claim 11 wherein ultrasonics are used to lyse bacterial cells.
14. A process according to claim 9, 11 or 13, wherein the cation exchange material is brought equally into contact with the gamma-interferon C-10L in order to achieve an equal distribution over the cation exchange material, and then it is washed and eluted in a batch method.
15. A process according to claim 9, 11, 13 or 14, wherein commonly used cation exchange materials are used.
16. A process according to claim 15, wherein the cation exchange material is CM-cellulose™ or Affi-Gel-Blue™.
17. Use of the polypeptide according to claim 1 in medicine as a therapeutic agent for the treatment of human lung and colon carcinoma cells, rheumatism and kidney cancer.
18. Use of the polypeptide according to claim 1 as a fine chemical for *in vitro* experiments wherein the polypeptide is used for interferon level measurements.

2096532

1 / 2

Figur 1

Protein and DNA sequence of human IFN-gamma variant C-10L

0
Met
ATG

1
GlnAspProTyrValLysGluAlaGluAsnLeuLysLysTyrPheAsnAlaGlyHisSer 20
CAAGACCCATATGTAAAAGAAGCAGAAAACCTTAAGAAATATTTAATGCAGGTCATTCA
179

AspValAlaAspAsnGlyThrLeuPheLeuGlyIleLeuLysAsnTrpLysGluGluSer 40
GATGTAGCGGATAATGGAACTCTTTTCTTAGGCATTTTGAAGAATTGGAAAGAGCAGAGT

AspArgLysIleMetGlnSerGlnIleValSerPheTyrPheLysLeuPheLysAsnPhe 60
GACAGAAAAATAATGCAGAGCCAAATTGTCTCTTTTACTTCAAACCTTTTAAAAACTTT

LysAspAspGlnSerIleGlnLysSerValGluThrIleLysGluAspMetAsnValLys 80
AAAGATGACCAGAGCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATGAATGTCAG

PhePheAsnSerAsnLysLysLysArgAspAspPheGluLysLeuThrAsnTyrSerVal 100
TTTTTCAATAGCACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAATTATTCGCTA

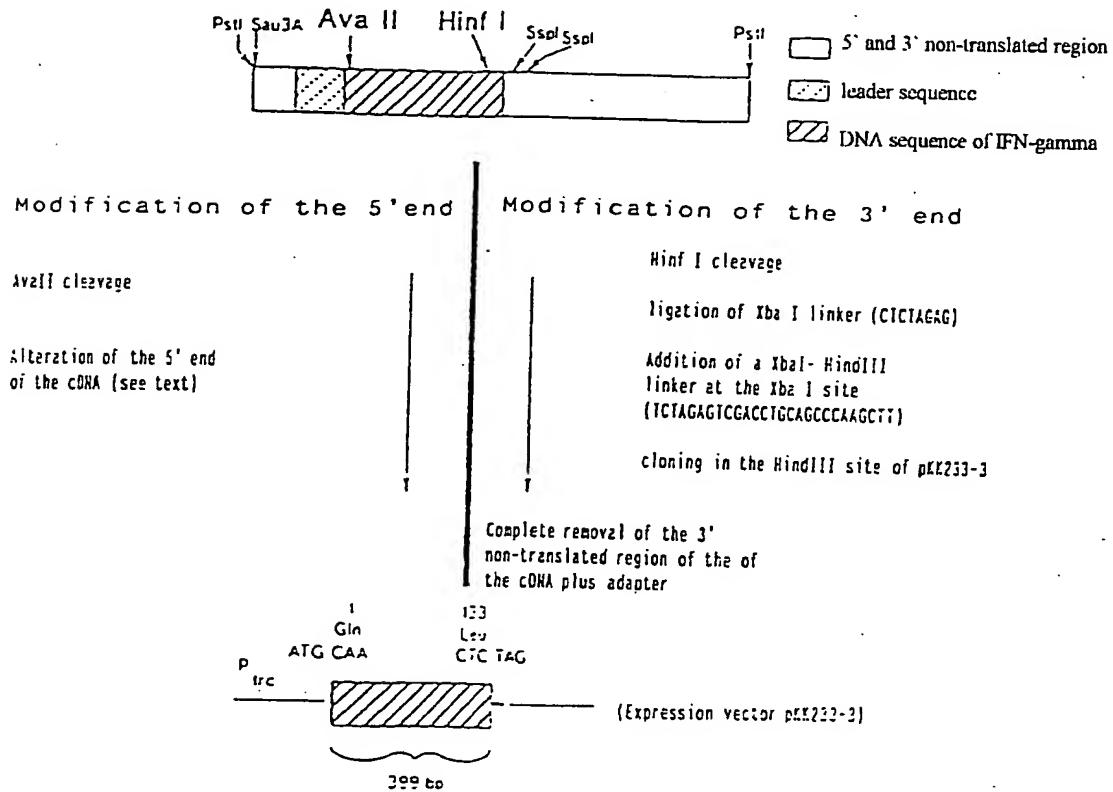
ThrAspLeuAsnValGlnArgLysAlaIleHisGluLeuIleGlnValMetAlaGluLeu 120
ACTGACTTGAAATGTCCAACGCAAGCAATACATGAACCTCATCCAAGTGATGGCTGAAC

SerProAlaAlaLysThrGlyLysArgLysArgSerLeu 133
TCGCCAGCAGCTAAAAACAGGGAAGCGAAAAAGGAGTCTC TAG
574

Figure 2

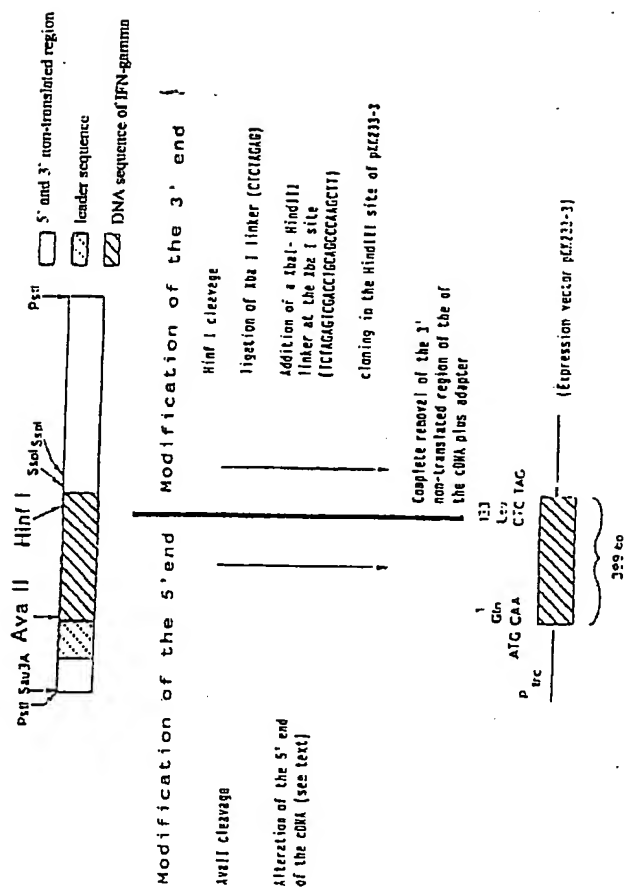
Construction of an expression plasmid for expression of
IFN-gamma C-10L

Human IFNg C-10L cDNA cloned in the PstI site of pBR322



Construction of an expression plasmid for expression of IFN-gamma C-10L

Human IFN γ C-10L cDNA cloned in the PstI site of pBR322



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.